

# Immunohistochemical Examination of Experimental *Streptococcus iniae* Infection in Japanese Flounder *Paralichthys olivaceus*

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**ABSTRACT**—To investigate the course of *Streptococcus iniae* infection in Japanese flounder *Paralichthys olivaceus*, fish (average weight 118±14 g) were experimentally infected by oral and bath methods, and the distribution and multiplication of *S. iniae* in the fish were monitored by bacteriological and immunohistochemical examinations. *S. iniae* was detected first in relatively high numbers in the kidney and spleen. Viable counts of *S. iniae* in the blood, brain, liver, stomach, intestine, gill, skin mucus and nares were high only when those in the kidney and spleen were high. *S. iniae*-laden phagocytic cells were observed in the lumen of the blood vessels distributing in the organs and tissues in the initial stage of infection. Many fish showed hemorrhagic lesions on the fins, and the extracellular multiplication of *S. iniae* in the hemorrhagic fins was observed in the initial stage of infection. These observations were common among the fish challenged by either method. There was no evidence of the entrance of *S. iniae* through the stomach, intestine, gill, eye or olfactory pouches of nares. It was presumed that *S. iniae* entered directly from the water through the abrasive sites of fins and was disseminated by the blood circulation to cause systemic infection.

**Key words:** *Streptococcus iniae*, *Paralichthys olivaceus*, experimental infection, streptococcosis, Japanese flounder, immunohistochemistry

Streptococcosis caused by *Streptococcus iniae* has been reported worldwide in many intensively cultured fish species. Natural and experimental *S. iniae* infections were described in rainbow trout *Oncorhynchus mykiss* (Kitao *et al.*, 1981), Japanese flounder *Paralichthys olivaceus* (Nakatsugawa, 1983), hybrid tilapia *Oreochromis niloticus* × *O. aureus* (Perera *et al.*, 1994), hybrid striped bass *Morone saxatilis* × *M. chrysops* (Stoffregen *et al.*, 1996), barramundi *Lates calcarifer* (Bromage *et al.*, 1999), rabbitfish *Siganus canaliculatus* (Yuasa *et al.*, 1999) and red drum *Sciaenops ocellatus* (Eldar *et al.*, 1999). Clinical signs of *S. iniae* infection are commonly characterized by darkness in color, disorientation in movements, uni- or bilateral exophthalmia, congestion of the fins and mouth, and, internally, congestion of the liver, spleen and kidney, and ascites.

Recently, various challenge methods of infection have been employed to study the route of infection of *S.*

*iniae*, such as immersion, oral administration, cohabitation with diseased fish and nares inoculation. Perera *et al.* (1997) suggested that *S. iniae* can cause disease in tilapia through water-borne as well as oral route of infection. Shoemaker *et al.* (2000) observed cannibalism of the eyes and viscera of moribund and dead fish in a cohabitation experiment and suggested oral and/or olfactory route of infection. Evans *et al.* (2000) were successful in experimental *S. iniae* infection of hybrid striped bass and tilapia by nares inoculation and suggested that nares may be a potential route of infection.

Japanese flounder, one of the important marine fish species cultured in Japan, has often been suffered from streptococcosis in high temperature months (Nakatsugawa, 1983). Our previous study demonstrated bath challenge was superior to oral challenge in induction of experimental infection in Japanese flounder (Nguyen *et al.*, 2001). Both challenge methods induced hemorrhagic lesions on the skin and fins; it is suggested that *S. iniae* may enter through the body surface such as abrasions. In the present study experimental oral and

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bath challenges were carried out to investigate the course of infection of *S. iniae* in Japanese flounder. Bacteriological and immunohistochemical techniques were used to monitor the sites of bacterial entry and the course of infection in fish at different time points after challenge. Using these techniques, the entry, distribution and multiplication of the bacterium in the fish, and their relationship to lesions can be more accurately followed.

## Materials and Methods

### Experimental infections

Experimental infections were carried out according to the previous report (Nguyen *et al.*, 2001). Flounder (average weight  $118 \pm 14$  g) were stocked into 200-L tanks for challenge groups of 30 fish each 1 d before challenge. Each tank was supplied with constant aeration and continuous water flow at approximately 6 L/min. The water temperature ranged from 25 to 26°C. The fish received no feed during the experiment.

*S. iniae* NUF631, originally isolated from a diseased flounder, was cultured on Todd-Hewitt (TH) agar plates at 28°C for 18 h, harvested by washing off the plates with sterile 10 mM phosphate-buffered saline (PBS), pH 7.2, to make bacterial suspension.

**Oral challenge:** Ground commercial pellet feed was mixed with an appropriate volume of the bacterial suspension at a ratio of 1(wt):2.5(v) to produce an injectable slurry. Each fish was anesthetized with tricaine methane sulfonate and received the slurry at a volume of 2% body weight (BW) by intragastric injection with a plastic catheter attached to a 10-mL syringe. Inoculation doses employed were  $2.0 \times 10^8$  and  $2.0 \times 10^9$  CFU/100 g BW.

**Bath challenge:** The water in the fish tanks was drained until the remaining was 10 L and then the bacterial suspension was poured in. Viable count of *S. iniae* in the water was confirmed by plate count on thallium acetate-oxolinic acid (TAOA) agar (duplicate) (Nguyen and Kanai, 1999). After 30 min of exposure, the tanks were filled in with water to reach the initial water level, and then the water change was commenced again. Inoculation doses employed were  $7.2 \times 10^6$  and  $7.2 \times 10^7$  CFU/mL water.

Dead fish were taken off daily and observed for gross lesions, and their brain and kidney were cultured on TH agar to confirm to be infected with *S. iniae*.

### Observation of the bacterial multiplication and progression of disease in experimentally infected flounder

Four fish were sampled randomly from each tank at five time points (6 and 24 h, and 3, 6 and 9 d) after challenge. Two were used for counting viable bacteria and others were subjected to immunohistochemical examination. In the bath challenge groups, however,

number of sampled fish at the later samplings was insufficient because of deaths of the fish due to the disease (see below).

**Viable count of *S. iniae* in tissue samples:** A small piece or volume of the brain, kidney, spleen, liver, stomach, intestine, gill, skin mucus and blood was ground by a glass homogenizer or vortexed with nine volumes of PBS to make suspension, from which serial tenfold dilutions were made. One hundred microliters of each dilution of the blood, brain, kidney, spleen and liver was plated on TH agar, and that of the stomach, intestine, gill and mucus was plated on TAOA blood (TAOAB) agar. A sample from nares was taken with a swab and the nasal swab was streaked on TAOAB agar. Inoculated plates were incubated at 28°C for 72 h. Colonies suspected to be those of *S. iniae* were counted, and among those several colonies were sub-cultured on TH agar and subjected to agglutination test with rabbit anti-*S. iniae* NUF631 serum to confirm to be *S. iniae*.

**Immunohistochemical detection of *S. iniae* in tissue samples:** Samples of the brain, kidney, spleen, liver, stomach, intestine, gill, eye, nares and hemorrhagic fins were fixed in 10% neutral buffered formalin, embedded in paraffin and then sectioned (3  $\mu$ m in thickness). Endogenous peroxidase activity in tissues was removed by incubating sections with 0.3% hydrogen peroxide in methanol for 30 min. Non-specific binding sites of antibody were blocked with 2% gelatin in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5; TBS). The blocked sections were incubated with rabbit anti-*S. iniae* NUF631 serum diluted 1:1,000 in 1% gelatin-Tween TBS (0.05% Tween 20 in TBS; TTBS) for 1 h at room temperature and then with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad; HRP-GAR) diluted 1:3,000 in 1% gelatin-TTBS for 30 min at room temperature. Specific bindings of HRP-GAR were detected by incubating with a mixture of 20 mg diaminobenzidine tetrahydrochloride (DAB) and 0.1 mL of 5% hydrogen peroxide in 100 mL of 50 mM Tris-HCl, pH 7.6, for 4 to 5 min. Sections were counter-stained with hematoxylin for 5 to 10 min. *S. iniae* was detected as brown color in a section against a bluish background. Rabbit anti-*Edwardsiella tarda* NUF49 serum and tissue samples obtained from healthy fish were used for negative control.

## Results

### Mortality of fish in the challenge experiment

Numbers of the dead flounder monitored at different time periods are shown in Table 1, although mortality rate was difficult to be calculated due to the thinning out of fish for sampling. Fish died first at the time period between 24 h and 3 d after challenge. Deaths occurred mainly between 3 and 6 d in all the challenge groups

**Table 1.** Number of dead Japanese flounder monitored at different time periods after challenge with *S. iniae* NUF631

Challenge method	Inoculation dose (CFU/100 g BW or mL water)	No. of dead fish at time period between					Total no. of death
		0–6 h	6–24 h	24 h–3 d	3–6 d	6–9 d	
Oral	$2.0 \times 10^8$	0	0	3	2	3	8
	$2.0 \times 10^9$	0	0	2	7	1	10
Bath	$7.2 \times 10^6$	0	0	5	10	*	15
	$7.2 \times 10^7$	0	0	5	8	0	13

\* No fish remained in the challenge group.

**Table 2.** Viable count (CFU/g or mL) of *S. iniae* from flounder in the oral challenge groups at time points

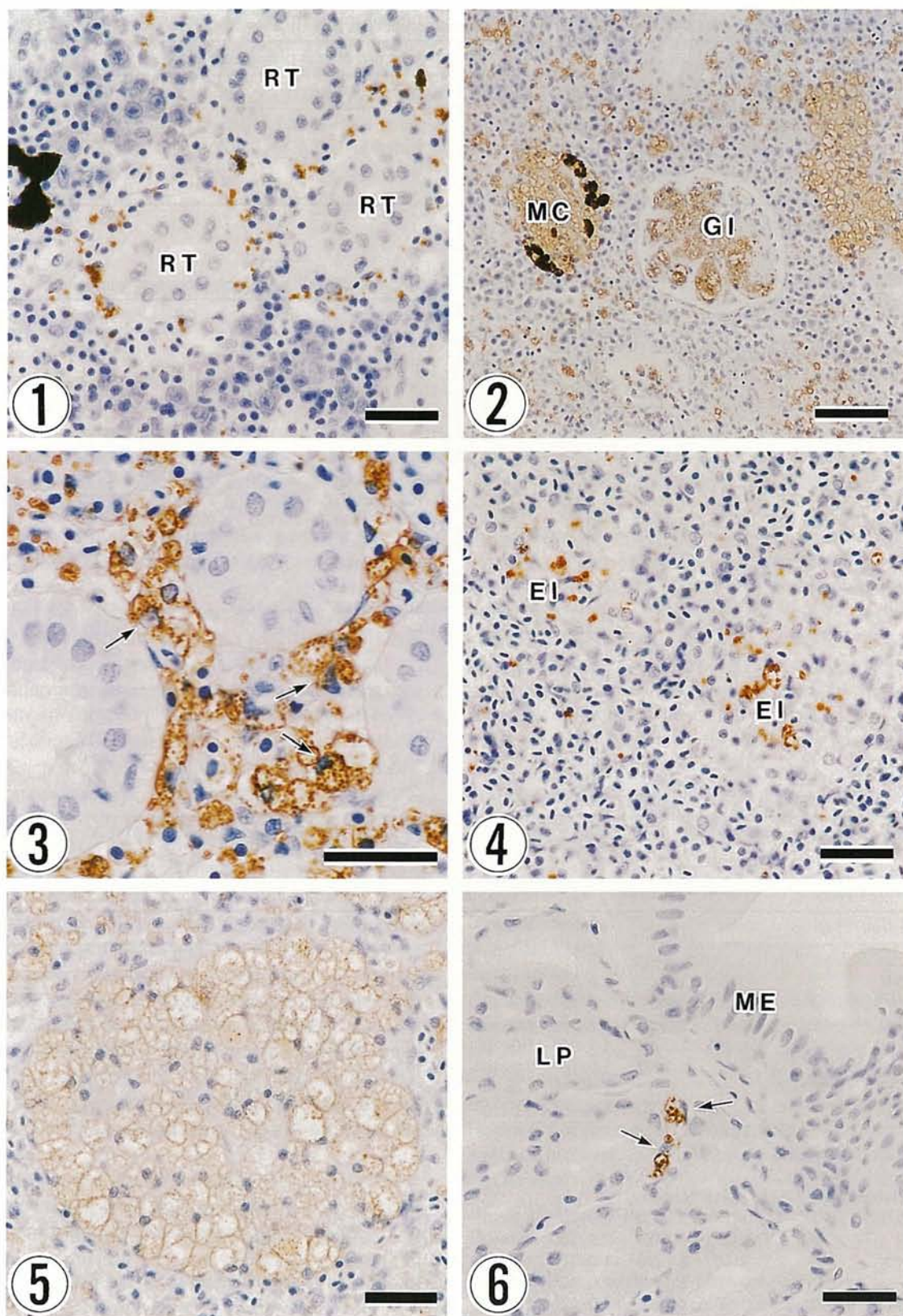
Time point	Fish no.	Blood	Brain	Kidney	Spleen	Liver	Stomach	Intestine	Gill	Skin mucus	Nasal swab*
Inoculation dose: $2.0 \times 10^8$ CFU/100 g BW											
6 h	1	<100	<100	<100	<100	<100	<100	$2.0 \times 10^7$	<100	<100	–
	2	<100	<100	<100	<100	<100	<100	$7.5 \times 10^6$	<100	<100	–
24 h	3	<100	<100	<100	<100	<100	<100	<100	$1.0 \times 10^2$	<100	–
	4	<100	<100	<100	<100	<100	<100	<100	<100	<100	–
3 d	5	<100	<100	<100	<100	<100	<100	<100	<100	$5.1 \times 10^3$	–
	6	$3.3 \times 10^7$	$3.0 \times 10^6$	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.4 \times 10^7$	$7.5 \times 10^5$	$2.0 \times 10^7$	$3.5 \times 10^7$	$5.0 \times 10^5$	+++
6 d	7	$2.5 \times 10^7$	$4.7 \times 10^7$	$3.4 \times 10^8$	$9.0 \times 10^7$	$2.0 \times 10^8$	$1.8 \times 10^7$	$1.8 \times 10^7$	$6.5 \times 10^7$	$7.3 \times 10^4$	+++
	8	<100	<100	<100	<100	<100	<100	<100	$7.3 \times 10^3$	$1.3 \times 10^3$	–
9 d	9	<100	<100	<100	<100	<100	$1.0 \times 10^2$	$1.0 \times 10^2$	$6.1 \times 10^3$	$2.9 \times 10^3$	+
	10	$6.0 \times 10^2$	<100	$5.2 \times 10^4$	$1.0 \times 10^4$	$7.0 \times 10^4$	<100	<100	$1.1 \times 10^4$	$1.2 \times 10^3$	+
Inoculation dose: $2.0 \times 10^9$ CFU/100 g BW											
6 h	1	<100	<100	<100	<100	<100	<100	$6.3 \times 10^8$	<100	<100	–
	2	<100	<100	<100	<100	<100	<100	$5.6 \times 10^8$	<100	<100	–
24 h	3	<100	<100	<100	<100	<100	<100	<100	$1.1 \times 10^3$	<100	–
	4	$1.2 \times 10^3$	$4.0 \times 10^2$	$1.2 \times 10^6$	$1.6 \times 10^6$	$2.0 \times 10^4$	$1.0 \times 10^2$	$1.2 \times 10^4$	$7.4 \times 10^3$	$3.0 \times 10^2$	–
3 d	5	$1.6 \times 10^7$	$1.7 \times 10^7$	$1.0 \times 10^9$	$1.0 \times 10^9$	$8.2 \times 10^7$	$8.8 \times 10^6$	$3.0 \times 10^7$	$2.4 \times 10^7$	$6.2 \times 10^5$	+++
	6	<100	<100	$6.0 \times 10^3$	$4.0 \times 10^3$	$1.9 \times 10^3$	$4.0 \times 10^2$	$1.0 \times 10^2$	$1.7 \times 10^3$	$4.0 \times 10^3$	–
6 d	7	<100	<100	<100	<100	<100	<100	$6.0 \times 10^2$	$1.4 \times 10^3$	$2.0 \times 10^2$	–
	8	<100	<100	<100	<100	<100	$2.0 \times 10^2$	$1.0 \times 10^2$	$3.2 \times 10^4$	$5.0 \times 10^2$	–
9 d	9	<100	<100	<100	<100	<100	<100	<100	<100	<100	–
	10	<100	<100	<100	<100	<100	<100	<100	$1.0 \times 10^2$	$1.0 \times 10^2$	–

\* Colony number on the agar plate from streaking nasal swab was graded at levels as –, 0; +, 1–30; ++, 31–300; +++, &gt;300.

**Table 3.** Viable count (CFU/g or mL) of *S. iniae* from flounder in the bath challenge groups at time points

Time point	Fish no.	Blood	Brain	Kidney	Spleen	Liver	Stomach	Intestine	Gill	Skin mucus	Nasal swab*
Inoculation dose: $7.2 \times 10^6$ CFU/mL water											
6 h	1	<100	$3.0 \times 10^2$	$8.8 \times 10^4$	$7.4 \times 10^4$	$2.0 \times 10^2$	$1.0 \times 10^2$	$1.0 \times 10^2$	$2.0 \times 10^2$	<100	+
	2	$7.6 \times 10^3$	$4.0 \times 10^2$	$1.0 \times 10^6$	$3.7 \times 10^5$	$4.1 \times 10^3$	$4.0 \times 10^2$	$4.6 \times 10^3$	$5.1 \times 10^3$	$1.4 \times 10^3$	+
24 h	3	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.0 \times 10^9$	$9.2 \times 10^7$	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.2 \times 10^7$	+++
	4	$4.8 \times 10^7$	$1.0 \times 10^6$	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.4 \times 10^7$	$2.0 \times 10^7$	$3.4 \times 10^7$	$2.5 \times 10^7$	$8.3 \times 10^5$	+++
3 d	5	$8.2 \times 10^7$	$2.0 \times 10^7$	$1.0 \times 10^9$	$1.0 \times 10^9$	$5.5 \times 10^7$	$1.1 \times 10^7$	$3.4 \times 10^7$	$3.7 \times 10^7$	$1.6 \times 10^5$	+++
	6	$5.5 \times 10^7$	$1.6 \times 10^7$	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.2 \times 10^7$	$8.7 \times 10^7$	$1.0 \times 10^9$	$3.8 \times 10^5$	+++
6 d	7	$2.4 \times 10^6$	$3.7 \times 10^7$	$1.5 \times 10^8$	$8.2 \times 10^7$	$8.1 \times 10^7$	$1.5 \times 10^7$	$3.9 \times 10^7$	$1.2 \times 10^7$	$6.8 \times 10^3$	+++
	8	$7.4 \times 10^5$	$4.0 \times 10^7$	$7.1 \times 10^7$	$3.1 \times 10^7$	$2.5 \times 10^7$	$1.3 \times 10^7$	$1.0 \times 10^7$	$4.7 \times 10^6$	$9.9 \times 10^3$	+++
9 d	No fish										
	No fish										
Inoculation dose: $7.2 \times 10^7$ CFU/mL water											
6 h	1	$3.0 \times 10^4$	$1.1 \times 10^3$	$2.0 \times 10^6$	$5.0 \times 10^5$	$4.8 \times 10^3$	$8.0 \times 10^2$	$5.0 \times 10^3$	$4.6 \times 10^3$	$8.0 \times 10^2$	+++
	2	<100	<100	$2.9 \times 10^3$	$2.8 \times 10^3$	<100	<100	$2.0 \times 10^3$	$2.0 \times 10^2$	$3.0 \times 10^2$	+
24 h	3	$5.3 \times 10^7$	$1.4 \times 10^6$	$1.0 \times 10^9$	$1.0 \times 10^9$	$2.1 \times 10^7$	$1.2 \times 10^7$	$8.2 \times 10^7$	$3.4 \times 10^6$	$4.2 \times 10^6$	+++
	4	$1.0 \times 10^9$	$5.6 \times 10^6$	$1.0 \times 10^9$	$1.0 \times 10^9$	$8.0 \times 10^7$	$1.7 \times 10^7$	$8.3 \times 10^7$	$1.2 \times 10^7$	$2.5 \times 10^6$	+++
3 d	5	$5.1 \times 10^7$	$1.3 \times 10^7$	$1.0 \times 10^9$	$1.0 \times 10^9$	$9.2 \times 10^7$	$2.5 \times 10^7$	$5.7 \times 10^7$	$2.5 \times 10^7$	$2.4 \times 10^5$	+++
	6	$3.6 \times 10^7$	$1.0 \times 10^7$	$1.0 \times 10^9$	$1.0 \times 10^9$	$7.4 \times 10^7$	$2.5 \times 10^6$	$4.4 \times 10^7$	$5.3 \times 10^7$	$4.0 \times 10^5$	+++
6 d	7	$4.1 \times 10^6$	$3.7 \times 10^7$	$1.4 \times 10^8$	$5.1 \times 10^7$	$8.1 \times 10^7$	$1.4 \times 10^7$	$3.5 \times 10^6$	$9.8 \times 10^6$	$6.5 \times 10^5$	+++
	8	$3.6 \times 10^5$	$2.1 \times 10^8$	$1.7 \times 10^7$	$1.1 \times 10^7$	$2.1 \times 10^7$	$3.7 \times 10^6$	$1.7 \times 10^7$	$3.1 \times 10^6$	$3.6 \times 10^4$	+++
9 d	9	$4.4 \times 10^7$	$3.6 \times 10^5$	$2.8 \times 10^7$	$2.0 \times 10^7$	$1.4 \times 10^7$	$1.7 \times 10^6$	$1.4 \times 10^7$	$8.0 \times 10^6$	$3.8 \times 10^3$	++
	No fish										

\* Colony number on the agar plate from streaking nasal swab was graded at levels as +, 0; +, 1–30; ++, 31–300; +++, &gt;300.



**Fig. 1.** An initial stage of infection in the kidney of Japanese flounder at 24 h after bath challenge. *Streptococcus iniae* is detected in the interstitial tissue surrounding the renal tubules (RT). Bar = 20  $\mu$ m.

**Fig. 2.** An advanced stage of infection in the kidney at 6 d after oral challenge. *S. iniae* expands into the glomeruli (GI), melanomacrophage center (MC) and wider in the interstitial tissue surrounding the renal tubules. Bar = 40  $\mu$ m.

**Fig. 3.** An advanced stage of infection in the kidney at 3 d after oral challenge showing enlarged and necrotic *S. iniae*-laden phago-



except for the oral challenge group with inoculation dose of  $2.0 \times 10^8$  CFU/100 g BW, in which fish died gradually and there remained two fish at the end of the experiment. Total numbers of deaths in the bath challenge groups were more than those in the oral challenge groups. *S. iniae* was recovered from both brain and kidney of all dead fish. Bacterial cultures from the brain and kidney of the two survivors were negative for *S. iniae*, and no clinical signs were observed in either fish.

#### Gross examination of the dead fish

Gross lesions observed in the dead fish were common among the fish challenged by either method. Gross examinations of the early deaths showed small hemorrhagic lesions on the dorsal and pectoral fins. Internally, mild swelling of the liver, spleen and kidney was observed. For those of the later deaths, gross lesions were more obvious. Severe hemorrhages of the fins extending to the proximal margins were seen in many fish. Some dead fish showed ocular abnormalities such as clouding of cornea and internal hemorrhagic exophthalmia, hemorrhages of the gills, inner surface of the opercula and abdominal wall, and ascites. The liver, spleen and kidney of these fish were swollen and pale. Gastric and intestinal lesions were not observed in any moribund or dead fish.

#### Viable counts of *S. iniae* in the tissues of sampled fish at time points

Viable counts of *S. iniae* in the tissues of sampled fish are shown in Tables 2 and 3. *S. iniae* was detected in relatively high numbers in the kidney and spleen in all the challenge groups. The viable counts in the blood, brain, liver, gill, skin mucus and nasal swab were high only when those in the kidney and spleen were high. All the fish in the bath challenge groups were infected with *S. iniae*. On the other hand, many fish in the oral challenge groups were devoid of the bacterium in the blood, brain, kidney, spleen and liver. The intestines of the fish examined at 6 h in the oral challenge groups still contained injected feed and the viable counts were high, while the stomachs of these fish were empty and the viable counts were low.

#### Status of *S. iniae* in the organs

A few *S. iniae*-laden phagocytic cells were occasionally observed in the lumen of the blood vessels distributing in various tissues in the initial stage of infection. In the advanced stages, profuse numbers of phagocytic cells laden with numerous bacteria were

observed, most of which were necrotized. Infiltration of these phagocytic cells to the parenchymal tissues surrounding the blood vessels or throughout the parenchyma resulted in the destruction of the parenchymal tissues. These findings were common in the fish challenged by either method and inoculation dose. Observations of *S. iniae* infection in different organs were described below.

**Kidney:** In the initial stage of infection *S. iniae*-laden phagocytic cells were seen in the small blood vessels adjacent to the renal tubules (Fig. 1). Sections of extensively infected kidneys showed *S. iniae* spreading into the glomeruli and melanomacrophage centers and wider in the interstitial tissues surrounding the renal tubules (Fig. 2). Most *S. iniae*-laden phagocytic cells were enlarged and necrotic due to intracellular bacterial multiplication (Fig. 3).

**Spleen:** In the initial stage of infection *S. iniae* and *S. iniae*-laden phagocytic cells were observed in the ellipsoids (Fig. 4). In the advanced stages of infection *S. iniae* was seen throughout the splenic stroma. Destruction of the ellipsoids due to extensive bacterial multiplication was observed (Fig. 5).

**Stomach and intestine:** *S. iniae* was initially detected in the lamina propria, submucosa and connecting tissues between muscularis layers but not in the mucosal epithelium. High magnification views showed *S. iniae*-laden phagocytic cells in the lumen of the blood vessels distributing in these tissues (Figs. 6 & 7). In the advanced stages of infection *S. iniae* expanded their distribution throughout the wall of the stomach and intestine including the mucosal epithelium (Figs. 8 & 9).

**Liver:** A few *S. iniae*-laden phagocytic cells were detectable in the lumen of sinusoids of the hepatic parenchyma in the initial stage of infection (Fig. 10). Liver sections of severely infected fish showed that the sinusoids were filled with necrotic *S. iniae*-laden phagocytic cells and *S. iniae* (Fig. 11).

**Heart:** *S. iniae* was detected in the epicardium and less frequently in the myocardium. Sections of extensively infected hearts showed that most *S. iniae*-laden phagocytic cells in the epicardium and vascular system of the myocardium were necrotized resulting in the occurrence of extracellular bacteria in these tissues (Fig. 12).

**Brain:** Infiltration of phagocytic cells was more frequently seen in the meninges than in the ventricles. Phagocytic cells laden with profusely multiplied *S. iniae* inside and extracellular *S. iniae* were seen in the meninges. However, the cortex of the brain remained

cytic cells (arrows) and extracellular *S. iniae* in the interstitial tissue surrounding the renal tubules. Bar = 20  $\mu$ m.

- Fig. 4.** An initial stage of infection in the spleen at 24 h after bath challenge. *S. iniae* is detected in the ellipsoids (EI). Bar = 20  $\mu$ m.  
**Fig. 5.** An advanced stage of infection in the spleen at 9 d after oral challenge showing the destruction of an ellipsoid due to extensive bacterial multiplication. Bar = 20  $\mu$ m.  
**Fig. 6.** An initial stage of infection in the stomach at 24 h after bath challenge. *S. iniae*-laden phagocytic cells (arrows) are observed in the lamina propria (LP) but not in the mucosal epithelium (ME). Bar = 20  $\mu$ m.

mostly unaffected (Fig. 13).

**Gills:** In the initiation of infection *S. iniae* was seen mostly in the first lamella. In the extensively infected gill sections *S. iniae* was more frequently detected in the secondary lamella. High magnification views showed *S. iniae*-laden phagocytic cells in the lumen of the blood vessels in the lamellas (Fig. 14).

**Eyeball:** The choroid in the posterior wall of the eyeball was the initial site in which *S. iniae*-laden phagocytic cells were often observed (Fig. 15). Sections of severely infected eyes showed a large number of *S. iniae*-laden phagocytic cells and extracellular *S. iniae* in the ocular cavity (Fig. 16). Infiltration of *S. iniae*-laden phagocytic cells was sometimes observed in the cornea.

**Olfactory pouches of nares:** *S. iniae*-laden phagocytic cells were often detected in the olfactory lamina propria of severely infected fish (Fig. 17).

**Hemorrhagic fins:** Massive amounts of extracellularly multiplied *S. iniae* were found together with infiltrated phagocytic cells and red blood cells (Fig. 18). Loss of the epidermis and necrosis of the dermal tissues of the skin adjacent to the bony girdle were observed. *S. iniae*-laden phagocytic cells and extracellular *S. iniae* were also observed in the lumen of the blood vessels in the hemorrhagic fins.

## Discussion

Clinical signs of the disease have been reported in natural or experimental *S. iniae* infections of various fish species. Eldar *et al.* (1999) suggested that difference in fish species and variation in environmental conditions might be responsible for the difference in pathological lesions. Moreover, disease signs may vary according to natural or experimental infection and among experimental methods (Evans *et al.*, 2000).

In this study, gross signs of the experimentally infected flounder varied with the time after challenge but not with the methods used to challenge fish. Gross examinations of the early deaths showed only small hemorrhagic lesions on the fins and skin and mild swellings of the liver, kidney and spleen. These deaths could be considered due to peracute infection probably caused by invasion of high numbers of bacteria at a time. The peracute form of *S. iniae* infection was characterized by a sudden death of fish and diagnosis was possible only by isolating *S. iniae* from infected organs (Eldar *et al.*, 1995). On the other hand, disease signs

such as ocular abnormalities, ascites and congestion of the visceral organs observed in the fish that died later were the typical signs of the disease.

Although four fish were sampled at each time for detecting *S. iniae*, the mortality in the bath challenge groups was higher than that in the oral challenge groups. Additionally, the detection of *S. iniae* in the blood, brain, kidney, spleen and liver of sampled fish showed that all the fish challenged by the bath method were infected, while many fish challenged by the oral method were devoid of the bacterium in these organs. This result was in agreement with those reported in our previous study (Nguyen *et al.*, 2001) where bath challenge induced disease more easily than oral challenge.

*S. iniae* infection was monitored in different organs after introducing the bacterium to fish through oral and bath challenge methods. The result of detection of *S. iniae* in the blood, brain, kidney, spleen and liver of sampled fish in the bath challenge groups at 6 h suggests that *S. iniae* spread in the body rapidly from the sites of entry. At 24 h after challenge higher viable counts were obtained in the blood and all organs tested. This suggests that bacterial multiplication occurred. On the other hand, in the oral challenge groups *S. iniae* was detected in the organs later at 24 h or 3 d according to inoculation dose. This might be due to a small number of *S. iniae* that entered the fish body.

Generally, *S. iniae*-laden phagocytic cells were observed in the lumen of the blood vessels distributing in tissues and organs examined in the initial stages of infection. This suggests that *S. iniae* spread throughout fish body via infected phagocytic cells. However, bacterial multiplication occurring within phagocytic cells induces necrosis of these cells. As a result, free bacterial cells are released and they also contribute to the dissemination processes.

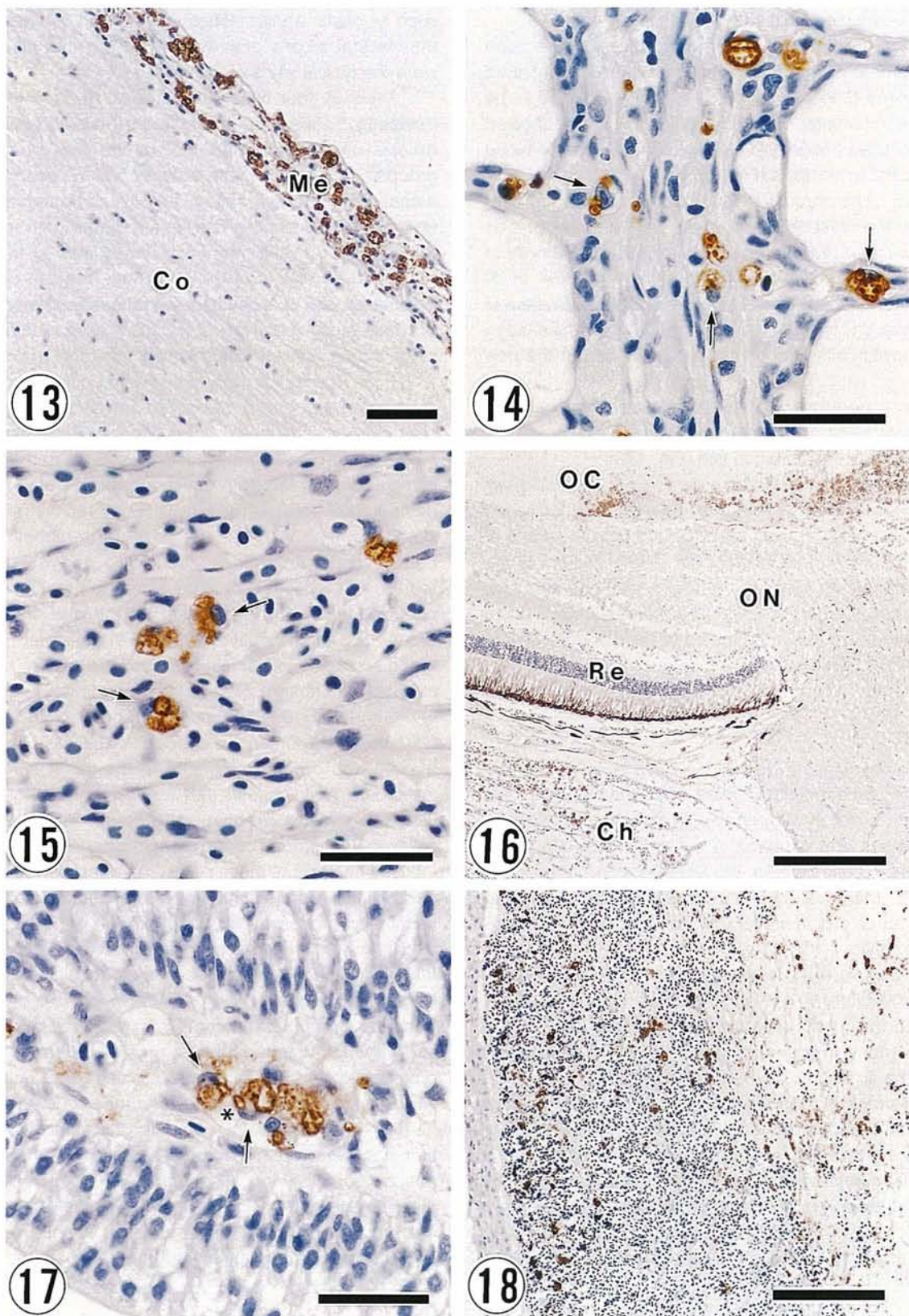
In this experiment inoculation doses employed were fairly high so that the degree of the bacterial multiplication in some tissues and organs might be different from that occurring in natural *S. iniae* infection. In the experimentally infected fish profuse *S. iniae*-laden phagocytic cells infiltrated the stomach and intestine, while few were observed in the cornea. On the other hand, in naturally infected flounder the extensive infection was observed in the cornea, but few *S. iniae*-laden phagocytic cells were observed in the blood vessels of the stomach and intestine (Nguyen, unpublished data). The absence of pathological changes in the intestine, and the bacterial invasion and infiltration of bacteria-

invades into the mucosal epithelium (arrows). Bar = 100  $\mu$ m.

**Fig. 10.** An initial stage of infection in the liver at 24 h after bath challenge. A few *S. iniae*-laden phagocytic cells (arrows) are observed in the lumen of sinusoid (asterisk). Bar = 10  $\mu$ m.

**Fig. 11.** An advanced stage of infection in the liver at 3 d after bath challenge. *S. iniae* is extensively detected in the lumen of the sinusoid (arrows) and the small vein (asterisk). Bar = 40  $\mu$ m.

**Fig. 12.** An infected heart at 6 d after bath challenge. Infiltration of *S. iniae*-laden phagocytic cells is observed in the epicardium (Ep) and myocardium (My). Bar = 40  $\mu$ m.



**Fig. 13.** An infected brain at 6 d after oral challenge. Infiltration of *S. iniae*-laden phagocytic cells is observed in the meninge (Me). The cortex (Co) remains unaffected. Bar = 40  $\mu$ m.

**Fig. 14.** An infected gill at 24 h after bath challenge. *S. iniae*-laden phagocytic cells (arrows) are observed in the lumen of the small blood vessels of the first and second lamella. Bar = 20  $\mu$ m.

**Fig. 15.** An initial stage of infection in the eye at 3 d after bath challenge. Infiltration of *S. iniae*-laden phagocytic cells (arrows) is



laden macrophages in the cornea were also observed in naturally *S. iniae*-infected tilapia (Miyazaki *et al.*, 1984; Perera *et al.*, 1998).

A large number of orally administrated *S. iniae* was recovered from the intestine at 6 h after challenge, but no *S. iniae* antigen was detected in the mucosal epithelium of the gastric folds or intestinal bulbs by immunohistochemical examinations. On the other hand, among the organs tested, the spleen and kidney were the first detectable sites of *S. iniae* infection. The occurrence of *S. iniae* in the tissues of the stomach and intestine was only evident after the spleen and kidney were extensively infected. Additionally, the infection in the stomach and intestine was initiated by the infiltration of *S. iniae*-laden phagocytic cells from the blood. These results implicate that the stomach and intestine are the secondary sites rather than the primary sites of infection.

Similarly, immunohistochemical examinations of the gills, eyes, olfactory pouches of sampled fish in the oral as well as bath challenge groups showed that the occurrence of *S. iniae* in these organs was only evident when the spleen and kidney were severely infected. Thus, the gills, eyes and olfactory pouches of nares could be considered as the secondary sites rather than the starting sites of *S. iniae* infection. Only in the hemorrhagic fins a great number of *S. iniae* was observed simultaneously with the initiation of infection in the spleen and kidney.

Streptococcosis caused by *S. iniae* has been described as a septicemic disease because of the isolation of the causative bacterium in the blood and various organs (Perera *et al.*, 1994, 1998; Yuasa *et al.*, 1999). It was observed that *S. iniae* multiplied extracellularly in the hemorrhagic fins. Immunohistochemical observation on the distribution of *S. iniae* in the spleen and kidney at the initiation of infection showed that the bacterium predominantly infected the interstitial tissues of the kidney and the ellipsoids in the spleen, where resident macrophages exist (Suzuki, 1995). It is supposed that part of *S. iniae* multiplying in the hemorrhagic fins may contact with phagocytic cells infiltrated from the blood. *S. iniae* and *S. iniae*-laden phagocytic cells enter the blood vessels and pass through the spleen and kidney where *S. iniae* will be taken by resident macrophages. After the multiplication in the kidney and spleen, *S. iniae* or *S. iniae*-laden macrophages enter the blood vessels again and distribute the other organs resulting in systematic infection. Occurrences of *S. iniae*-laden phagocytic cells in the capillary vessels of

various organs in the initial stage of infection support this supposition.

The absence of evidences that *S. iniae* entered through the mucosal epithelium of the stomach or intestine could not support the notion that the oral route of infection had occurred in the experiment. Based on the pathological and immunohistochemical observations of hemorrhagic lesions on the fins, it was strongly indicated that *S. iniae* entered through the body surface such as abrasive sites of fins.

## References

- Bromage, E. S., A. Thomas and L. Owens (1999): *Streptococcus iniae*, a bacterial infection in barramundi *Lates calcarifer*. *Dis. Aquat. Org.*, **36**, 177–181.
- Eldar, A., Y. Bejerano, A. Livoff, A. Horovitz and H. Bercovier (1995): Experimental streptococcal meningo-encephalitis in cultured fish. *Vet. Microbiol.*, **43**, 33–40.
- Eldar, A., S. Perl, P. F. Frelie and H. Bercovier (1999): Red drum *Sciaenops ocellatus* mortalities associated with *Streptococcus iniae* infection. *Dis. Aquat. Org.*, **36**, 121–127.
- Evans, J. J., C. A. Shoemaker and P. H. Klesius (2000): Experimental *Streptococcus iniae* infection of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) and tilapia (*Oreochromis niloticus*) by nares inoculation. *Aquaculture*, **189**, 197–210.
- Kitao, T., T. Aoki and R. Sakoh (1981): Epizootic caused by  $\beta$ -haemolytic *Streptococcus* species in cultured freshwater fish. *Fish Pathol.*, **15**, 301–307.
- Miyazaki, T., S. Kubota, N. Kaige and T. Miyashita (1984): A histopathological study of streptococcal disease in tilapia. *Fish Pathol.*, **19**, 167–172.
- Nakatsugawa, T. (1983): A streptococcal disease of cultured flounder. *Fish Pathol.*, **17**, 281–285. (In Japanese.)
- Nguyen, H. T. and K. Kanai (1999): Selective agars for the isolation of *Streptococcus iniae* from Japanese flounder, *Paralichthys olivaceus*, and its cultural environment. *J. Appl. Microbiol.*, **86**, 769–776.
- Nguyen, H. T., K. Kanai and K. Yoshikoshi (2001): Experimental *Streptococcus iniae* infection in Japanese flounder *Paralichthys olivaceus*. *Fish Pathol.*, **36**, 40–41.
- Perera, R. P., S. K. Johnson, M. D. Collins and D. H. Lewis (1994): *Streptococcus iniae* associated with mortality of *Tilapia nilotica* × *T. aurea* hybrids. *J. Aquat. Anim. Health*, **6**, 335–340.
- Perera, R. P., S. K. Johnson and D. H. Lewis (1997): Epizootiological aspects of *Streptococcus iniae* affecting tilapia in Texas. *Aquaculture*, **152**, 25–33.
- Perera, R. P., R. A. Fiske and S. K. Johnson (1998): Histopathology of hybrid tilapias infected with a biotype of *Streptococcus iniae*. *J. Aquat. Anim. Health*, **10**, 294–299.
- Shoemaker, C. A., J. J. Evans and P. H. Klesius (2000): Density and dose: factors affecting mortality of *Streptococcus iniae* infected tilapia (*Oreochromis niloticus*). *Aquaculture*, **188**,

observed in the choroid. Bar = 20  $\mu$ m.

**Fig. 16.** An advanced stage of infection in the eye at 6 d after oral challenge. *S. iniae* is detected in the ocular cavity (OC) and choroid (Ch). The retina (Re) and optic nerve (ON) remain unaffected. Bar = 200  $\mu$ m.

**Fig. 17.** An infected olfactory pouch at 3 d after bath challenge. *S. iniae*-laden phagocytic cells (arrows) are observed in the lumen (asterisk) of the small blood vessel in the lamina propria. Bar = 20  $\mu$ m.

**Fig. 18.** The adjacent skin of a hemorrhagic fin at 3 d after bath challenge. Hemorrhagic inflammation and extracellular multiplication of *S. iniae* are observed in the dermal tissue. Bar = 200  $\mu$ m.



- 229–235.
- Stoffregen, D. A., S. C. Backman, R. E. Perham, P. R. Bowser and J. G. Babish (1996): Initial disease report of *Streptococcus iniae* infection in hybrid striped (sunshine) bass and successful therapeutic intervention with the fluoroquinolone antibacterial enrofloxacin. *J. World Aquacult. Soc.*, **27**, 420–434.
- Suzuki, Y. (1995): Chapter VI. Vascular system, In “An atlas of fish histology: normal and pathological features” (ed. by F. Takashima and T. Hibiya). Kodansha, Tokyo, pp. 72–87.
- Yuasa, K., N. Kitanchaoen, Y. Kataoka and F. A. Al-Muraby (1999): *Streptococcus iniae*, the causative agent of mass mortality in rabbitfish *Siganus canaliculatus* in Bahrain. *J. Aquat. Anim. Health*, **11**, 87–93.